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14. ABSTRACT <p>The overall goal of this work is to engineer "synthetic probiotics": orally-administered gut bacteria that sense and compute the metabolic signature of a specific disease in the colon, and respond by secreting a therapeutic compound that treats the molecular basis of the disease. Here, we combined computation and experiments to discover the first known biological sensor of thiosulfate, a two-component system from <i>S. halifaxensis</i>. We transferred this sensor into the gut-adapted strain <i>E. coli</i> Nissle 1917 and used it to control GFP expression. We then administered this sensor strain to healthy mice and mice with colitis, and demonstrated that our sensor bacteria reliably report colitis. This work is a major advance toward our vision.</p>					
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Final Technical Report

Grant number: ONR N00014-14-1-0487

Title: Engineering probiotics that improve warfighter performance by maintaining lean body mass and inhibiting anxiety

Principal Investigator: Jeffrey J. Tabor

Institution: Rice University

Dates: May 15, 2014 – May 14, 2017

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Accomplishments

The major goal of this project was to engineer gut-adapted bacteria that sense molecular signature of a dysbiotic colon state, which is linked to inflammation (colitis), obesity, and anxiety, and respond by secreting molecules that prevent these disease phenotypes in mouse models.

We made several major advances toward this goal.

Computational mining of novel two-component system sensors

The first step in our synthetic probiotic approach is to engineer bacteria to sense gut metabolites linked to disease. However, synthetic biologists have developed very few well-characterized bacterial sensors. Thus, new methods for developing bacterial sensors of gut metabolites are needed.

Bacterial two-component systems (TCSs) are an exceptionally large and diverse family of biological sensors. The prototypical TCS is comprised of a membrane bound sensor histidine kinase (SK) and cytoplasmic response regulator (RR) that activates gene expression from an output promoter. Though tens of thousands of TCSs have been computationally identified, only a tiny fraction have been experimentally characterized.

We developed a novel method to discover novel TCS sensors of inputs of interest (e.g. chemical ligands, gut metabolites). In particular, we search all publically available DNA sequences for non-redundant SK genes with a standard Hidden Markov Model algorithm. We can focus our search on SKs with particular sensor domains that we believe likely to respond to our input of interest. For every identified SK, we then search for an adjacent RR same genome – a signature of a *bona fide* TCS pathway. We then analyze the nearby genome sequences to identify TCSs that reside adjacent to genes or gene clusters that are known to utilize, interact with, or respond to the input of interest, a signature that the TCS senses that input. Finally, we commercially synthesize the SK and RR genes and putative output promoter sequence (intergenic DNA region residing upstream of the presumptive output genes) and express the pathway under control of synthetic promoters and ribosome binding sites in standard chassis bacteria such as *E. coli*. Then, we perform a series of preliminary control experiments to demonstrate that the SK and RR are expressed, that the SK phosphorylates and/or dephosphorylates the RR, and that RR activates the output promoter. Finally, we screen the pathway for a specific response to our input of interest. Once, verified, we utilize the new system as a sensor. This method can be broadly applied to increase the number of sensors in

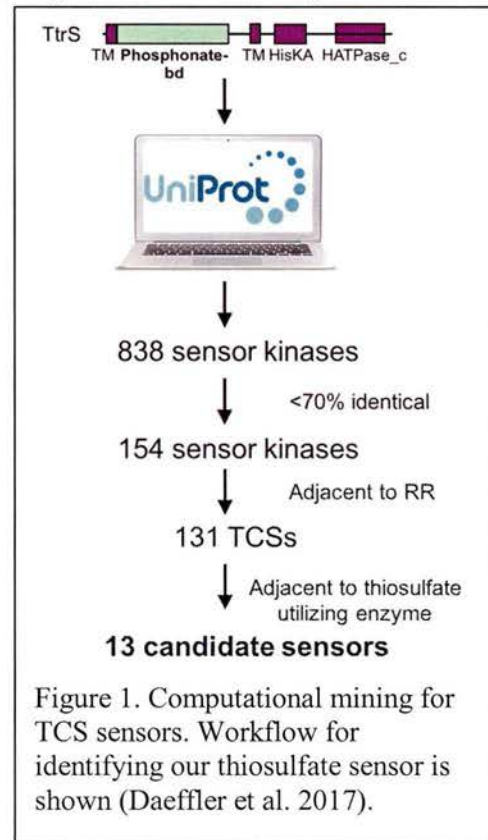


Figure 1. Computational mining for TCS sensors. Workflow for identifying our thiosulfate sensor is shown (Daeffler et al. 2017).

synthetic biology.

Discovery of novel thiosulfate and tetrathionate sensors

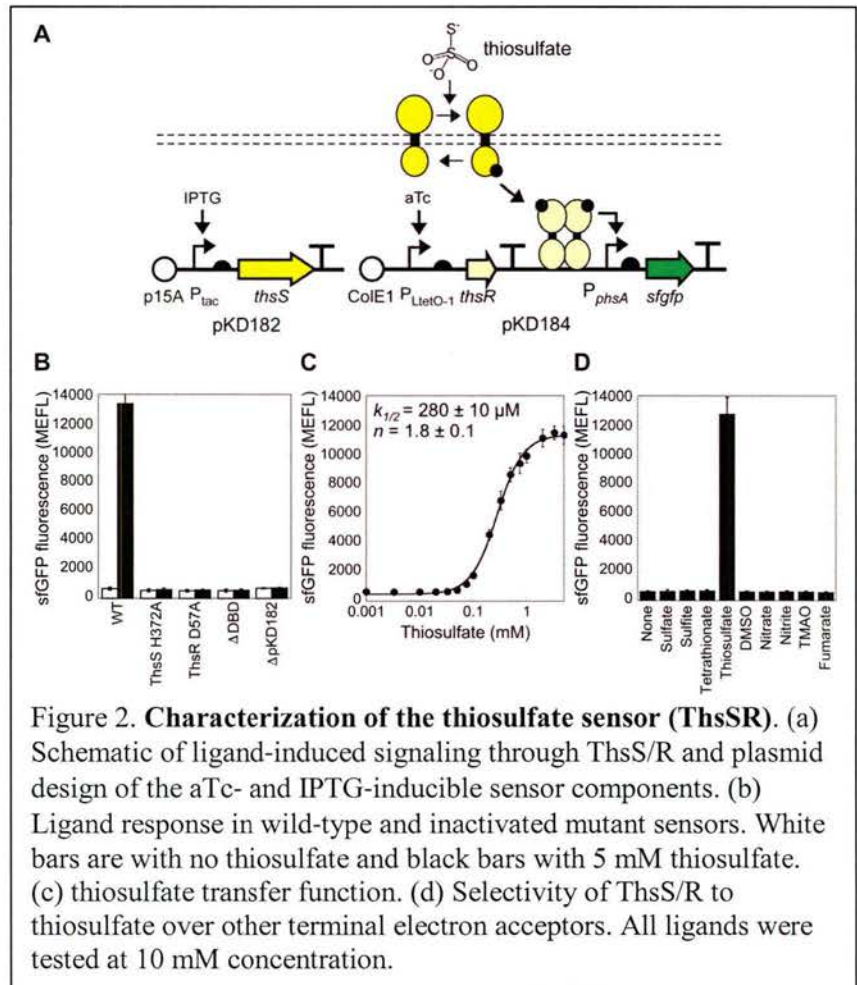
We utilized this method to discover the first known biological sensor of thiosulfate ($\text{S}_2\text{O}_3^{2-}$), a gut metabolite that we hypothesized is to be a biomarker of colitis, in the genome of the marine bacterium *Shewanella halifaxensis*. We also used the method to discover a novel sensor of tetrathionate ($\text{S}_4\text{O}_6^{2-}$), a gut metabolite previously linked to colitis, with improved performance features relative to a previously reported system. This second sensor comes from *Shewanella baltica*, another marine bacterium. Our results show that these *Shewanella* species likely use these sensors to induce expression of reductases to respire thiosulfate and tetrathionate, respectively.

Engineering colitis-sensing gut bacteria

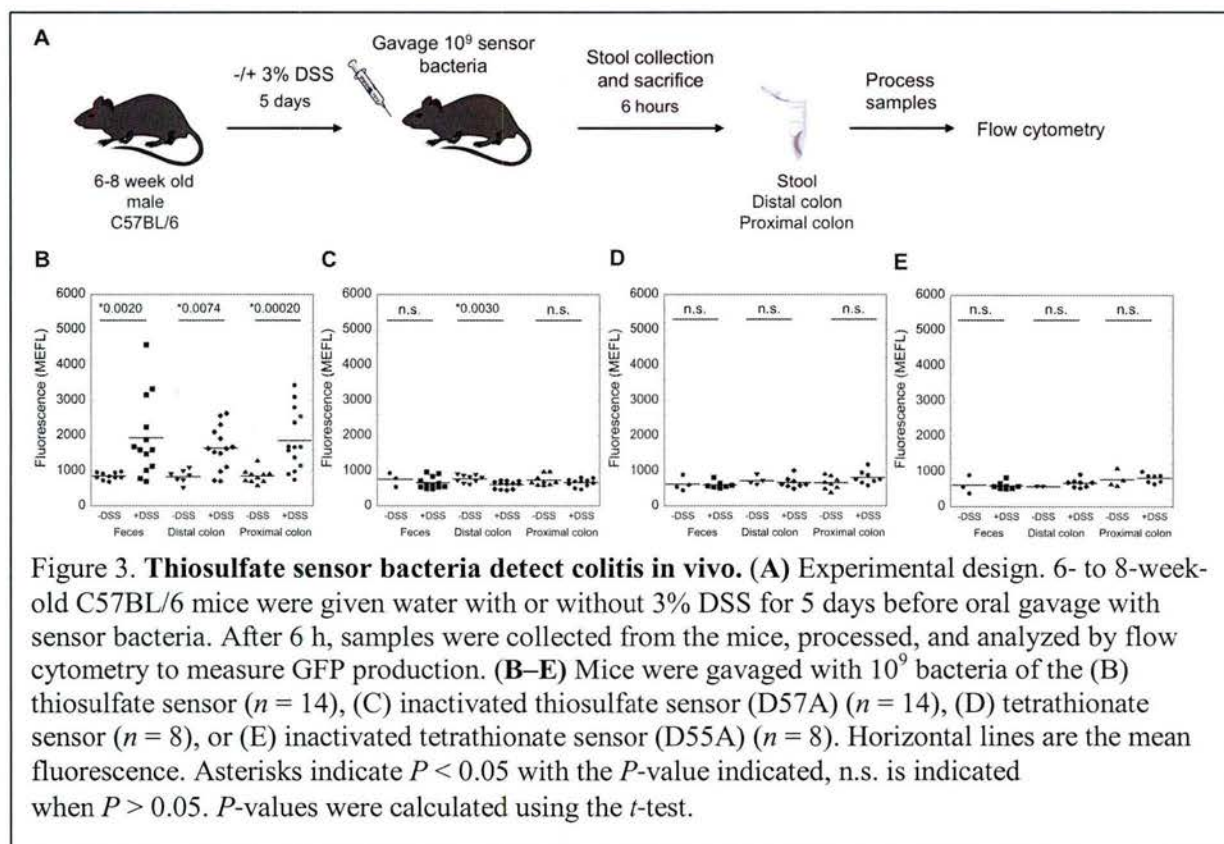
We then ported our thiosulfate and tetrathionate sensors into the gut-adapted strain *E. coli* Nissle 1917, and used them to control the expression of a GFP reporter gene. We co-expressed an mCherry gene to enable identification of our sensor strains amongst the complex gut microbiota. Then, we orally gavaged these sensor bacteria into healthy mice and mice treated with Dextran Sodium Sulfate (DSS) to induce colitis. Six hours later we harvested the fecal pellets and the contents of the colon. We then filtered the samples to purify our bacteria, stopped new GFP expression with an antibiotic, and allowed all GFP produced *in vivo* to

mature in the presence of atmospheric oxygen. Then, we identified our sensor bacteria and quantified their GFP output via flow cytometry. Finally, we used two blinded histopathologists to quantify the extent of colon inflammation in each animal.

As expected, our thiosulfate sensor is OFF in all healthy mice tested, but clearly and statistically significantly activated in mice with inflammation. Furthermore, the greater the extent of inflammation, the larger the GFP response. Interestingly, our tetrathionate sensor is inactive in



both healthy and inflamed mice. Thiosulfate is a low-priority respiratory electron acceptor and is thus thought to be stable once produced by the colon inflammatory pathway. On the other hand, tetrathionate is a high-priority electron acceptor and thought to be rapidly consumed by gut microbiota after production via the same pathway. Thus, we believe that thiosulfate is a superior colitis biomarker compared to tetrathionate.



Improving TCS sensors by DNA binding domain swapping

Many TCS output promoters are cross-regulated by alternative (often unknown) inputs that can result in false-negatives or false-positives *in vivo*. Additionally, many TCS output promoters are “silent” when ported to a new species, preventing sensor engineering.

We have developed a new method, whereby we modularly swap the DNA binding domain (DBD) of TCSs to rewire them to synthetic output promoters. We have shown that this method overcomes unwanted cross-regulation and unsilences TCSs when porting between species. We have used DBD-swapping to engineer sensors of the colitis-linked compounds nitrate (NO_3^-), and trimethyl amine *N*-oxide (TMAO).

Genetic circuit design

We aim to examine their efficacy in sensing colitis in future work. If they do sense colitis, we aim to combine them with the thiosulfate sensor via a 3-input AND gate to decrease false-positive diagnoses. To this end, we have developed a method whereby we can use CRISPR

interference (CRISPRi) to engineer such large genetic circuits – an important advance as CRISPRi systems have thus far been limited in size.

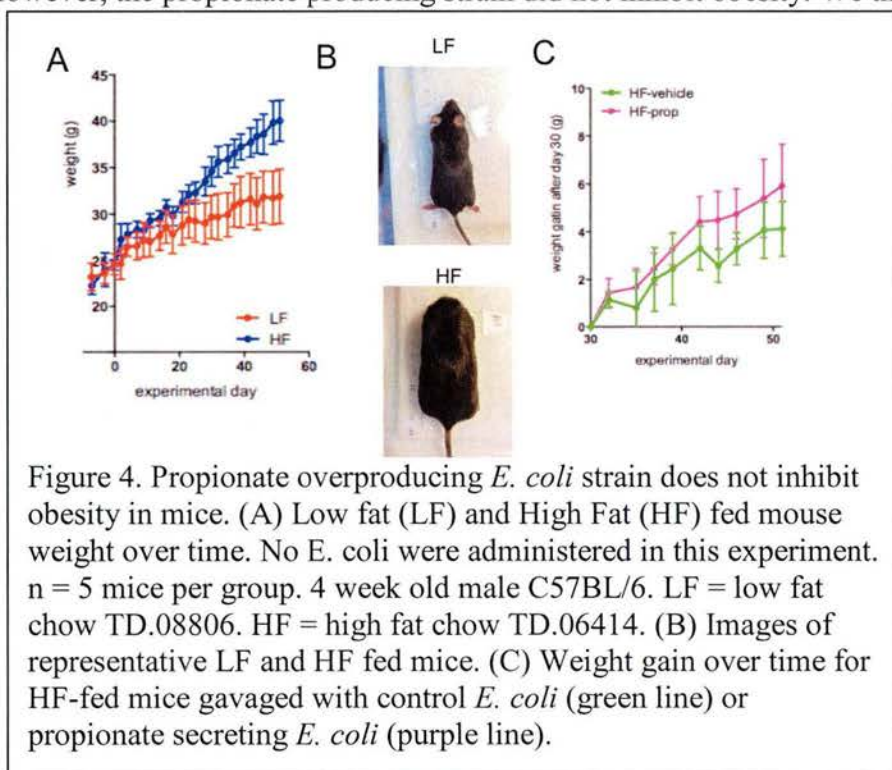
TCS tuning

TCS sensors are taken from nature. Thus, they have evolved to sense their inputs at concentration thresholds that are relevant to their host bacteria in a particular environment. However, these detection thresholds may be too low or high for synthetic biology applications including gut sensing, compromising their utility.

SKs dephosphorylate RRs in the absence of input. Here, we have demonstrated for the first time, that phosphatase activity increases TCS detection thresholds. Additionally, we have shown that introducing SK mutations that reduce phosphatase activity reduces detection threshold. Finally, we have identified a phosphatase “hot-spot” residue that can be mutated to tune phosphatase activity over a wide range of values. This “TCS tuning” method will enable the engineering of tailor-made sensors for a wide range of synthetic biology applications including engineering diagnostic and therapeutic gut bacteria.

Attempts to inhibit obesity

The short chain fatty acid propionate has been reported to increase satiety, decrease food consumption, and decrease obesity. The Prather group at MIT has previously engineered laboratory *E. coli* to secrete high levels of propionate by introducing a multi-gene metabolic pathway. The Prather group kindly shared this strain with us to test its inhibitory effect on obesity. We orally gavaged these bacteria, and negative control bacteria lacking the propionate production pathway, into mice eating low fat and high fat chow over several weeks and measured food intake and weight gain. Mice eating the high fat chow indeed exhibit weight gain and obese phenotypes. However, the propionate producing strain did not inhibit obesity. We then performed metabolic profiling on the fecal samples of these mice and observed that propionate levels were not elevated in the mice treated with the propionate secreting strain. It appears that the pathway is not active *in vivo*, possibly due to the fact that the strain was optimized for laboratory growth rather than gut growth. We have identified several alternative bacterial pathways to inhibit obesity, and the co-morbid phenotypes



of inflammation and anxiety. We hope to explore these further in future work, and link them to the sensors developed here.

Training and Professional Development

One postdoctoral scholar, Kristina Daeffler, was trained on this project. She developed the thiosulfate, tetrathionate, and TMAO sensors and demonstrated colitis sensing *in vivo*. She presented her results to the synthetic biology community at the 2015 SEED conference via a poster presentation and the 2016 SEED conference via a lightning talk and poster presentation.

Four Ph.D. students, Brian Landry, John Sexton, Felix Ekness, and Kathryn Brink were trained as part of this project. Brian developed the nitrate sensor and developed the TCS detection threshold tuning method. John Sexton engineered the CRISPRi circuits. Felix and Kathryn worked on the DBD swapping method. Brian presented his work via poster at the 2016 EBRC meeting at Northwestern University. John orally presented his work at the ACM NanoComm meeting in New York in 2016. Felix orally presented his work and Kathryn presented her work as a poster at the 2017 EBRC meeting at Georgia Tech.

Dissemination of results

Peer-reviewed journal publications

1. Landry, B.P., Palanki, R., Dyulgyarov, N., Hartsough, L.A., and **Tabor, J.J.*** Phosphatase activity tunes two-component system sensor detection threshold. *Submitted*.
2. Schmidl, S.R., Ekness, F., Sofjan, K., Daeffler, K.N-M., Brink, K., Landry, B.P., Dyulgyarov, N., Sheth, R.U., **Tabor, J.J.***. Response regulator DNA binding domain swapping enables discovery of bacterial two-component system inputs. *In revision*.
3. Landry, B.P., and **Tabor, J.J.***. Engineering diagnostic and therapeutic gut bacteria. *Microbiology Spectrum*. 2017; 5(5):BAD-0020-2017. doi:10.1128/microbiolspec.BAD-0020-2017.
4. Daeffler, K.N.M., Galley, J.D., Sheth, R.U., Ortiz-Velez, L.C., Shroyer, N.F., Britton, R.A., **Tabor, J.J.*** Engineering bacterial thiosulfate and tetrathionate sensors for detection of gut inflammation. *Molecular Systems Biology*. 2017 Apr 3;13(4):923. [PDF](#).
5. Castillo-Hair, S., Sexton, J., Landry B., Olson E.J., Igoshin, O.A, and **Tabor, J.J.***. FlowCal: A user-friendly, open source software tool for automatically converting flow cytometry data from arbitrary to calibrated units. (2016). *ACS Synthetic Biology*. 2016 Jul 15;5(7):774-80. [PDF](#).
6. Castillo-Hair, S., Igoshin, I., **Tabor J.J.***. How to train your microbe: tools for dynamically characterizing gene networks. *Current Opinion in Microbiology*. 2015 Apr;24:113-23. [PDF](#)

Conference papers

1. Sexton, J.T., and **Tabor, J.J.*** Multiplexed Bacterial Cell-Cell Communication via a Genetically Encoded CRISPRi-Based Multiplexer-Demultiplexer Circuit. ACM NanoCom. New York, NY. September 28-30, 2016. doi: 10.1145/2967446.2970372.

Presentations by PI Tabor

1. "Repurposing bacterial two-component systems for synthetic biology applications". Biochemistry Department. Michigan State University. East Lansing, MI. 09/23/17.
2. "Bacterial two-component systems as engineered sensors for synthetic biology". Sainsbury Laboratory Seminar Series, University of Cambridge, Cambridge, UK. 07/26/17.
3. "Engineering gut bacteria to diagnose disease". Bionetworks REU seminar. Rice University. Houston, TX. 06/14/17.
4. "Rewiring the DNA binding domains of bacterial two-component system response regulators" Molecular Biology of Infectious Diseases retreat. UT Health Science Center. Houston, TX. 03/31/17.
5. "Tuning the sensitivity of bacterial two-component sensors by mutation-driven modulation of phosphatase activity". Engineering Biology Research Center (EBRC) retreat. Evanston, IL. 03/25/17.
6. "Modularly rewiring DNA binding domains of bacterial two-component system response regulator proteins". School of Bioengineering. East China University of Science and Technology. Shanghai, China. 11/30/16.
7. "Modularly rewiring DNA binding domains of bacterial two-component system response regulator proteins". Cold Spring Harbor Asia Synthetic Biology Meeting. Suzhou, China. 11/29/16.
8. "Modularly rewiring DNA binding domains of bacterial two-component system response regulator proteins". Networks Seminar. University of Houston. Houston, TX. 11/18/16.
9. "Modularly rewiring DNA binding domains of bacterial two-component system response regulator proteins". 2016 ACS Southwest Regional Meeting (SWRM). Galveston, TX. 11/10/16.
10. "Engineering bacterial thiosulfate and tetrathionate sensors for detection of gut inflammation". International Workshop. New Horizons in Inflammation and Synthetic Biology. Harvard T.H. Chan School of Public Health. Boston, MA. 11/9/16.
11. "Modularly rewiring DNA binding domains of bacterial two-component system response regulator proteins". Center for Metagenomics and Microbiome Research Seminar Series. Baylor College of Medicine. 10/26/16.
12. "Rewiring the DNA binding domains of bacterial two-component system response regulators". Synthetic Biology, Engineering, Evolution and Design (SEED) conference. Chicago, IL. 07/20/16.
13. "Engineering bacterial two component sensors of gut inflammation". Microbiome for Health and Human Performance Workshop, Air Force Research Laboratory, Dayton, OH. 04/11/16.
14. "Engineering bacterial two component systems as sensors for synthetic biology". UC-Davis, Genome Center. 11/17/15.

15. "Engineering bacterial two component signaling systems as cellular sensors with high modularity, tunability, portability, and signal-to-background". UC-Berkeley, Structural and Quantitative Biology Seminar Series. 11/16/15.
16. "Engineering bacterial two component signal transduction systems to function as sensors of gut inflammation". Keck Seminar. Rice University. 09/18/15.
17. "Synthetic Probiotics". Computational and Integrative Biomedical Research (CIBR) Center, Baylor College of Medicine. Houston, TX. 11/20/14.

Awards

NSF CAREER (2016)

Technology Transfer

1. **Tabor, J.J.**, Landry, B.P., Dyulgyarov, N. PCT/US16/52281. Nitrate biosensor. Pending, Filed 09/16/16.
2. **Tabor, J.J.**, Daeffler K.N.M, Sheth, R.U. PCT/US16/52138. Thiosulfate biosensor for gut inflammation. Pending. Filed 09/16/16.
3. **Tabor, J.J.**, Schmidl, S., Sheth, R.U., Landry, B., Dyulgyarov, N. PCT/US16/30831. Identifying ligands from bacterial sensors. Pending. Filed 05/04/16.

Participants

First name: Jeffrey

Middle Name: J.

Last Name: Tabor

Email address: jeff.tabor@gmail.com

Most senior project role: PI

Nearest person month worked: 3

Contribution to the project: Manage all research.

Funding Support: 3 months

Countries of foreign collaborator: N/A

First name: Kristina

Middle Name: N.

Last Name: Daeffler

Email address: knmccleary@gmail.com

Most senior project role: Postdoctoral Researcher

Nearest person month worked: 35

Contribution to the project: Engineer TCSs, colitis sensing

Funding Support: 19 months

Countries of foreign collaborator: N/A

First name: John

Middle Name: T.

Last Name: Sexton

Email address: sexton.john.t@gmail.com

Most senior project role: Graduate Research Assistant

Nearest person month worked: 36
Contribution to the project: CRISPRi circuit design
Funding Support: 9 months
Countries of foreign collaborator: N/A

First name: Brian
Middle Name: P.
Last Name: Landry
Email address: brian.landry@gmail.com
Most senior project role: Graduate Research Assistant
Nearest person month worked: 36
Contribution to the project: TCS tuning, nitrate sensor
Funding Support: 2 months
Countries of foreign collaborator: N/A

First name: Felix
Middle Name:
Last Name: Ekness
Email address: felixekness@gmail.com
Most senior project role: Graduate Research Assistant
Nearest person month worked: 9
Contribution to the project: DBD swapping
Funding Support: 9 months
Countries of foreign collaborator: N/A

First name: Kathryn
Middle Name:
Last Name: Brink
Email address: krb6@rice.edu
Most senior project role: Graduate Research Assistant
Nearest person month worked: 3
Contribution to the project: DBD swapping
Funding Support: 3 months
Countries of foreign collaborator: N/A

Students:
Number of undergraduate STEM participants: 4

Number of graduate STEM participants: 4

Number of participants that received a STEM degree: 2